Strategies for immunohistochemical protein localization using antibodies: what did we learn from neurotransmitter transporters in glial cells and neurons

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Title: Strategies for immunohistochemical protein localization using antibodies: what did we learn from neurotransmitter transporters in glial cells and neurons

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Main Points:

Use of immunochemical methods without sufficient controls for specificity results in erroneous localization data.

We propose a strategy for protein localization and explain why quantitative considerations are important for specificity assessment.

Keywords: Antibody specificity, Immunocytochemistry, Western blotting, Glutamate uptake, Excitatory amino acid transporter

ABBREVIATIONS

The abbreviations used are: BGT1; betaine-GABA transporter (slc6a12); DTT, dithiothreitol; EAAT1, glutamate aspartate transporter (GLAST; slc1a3); EAAT2, glutamate transporter 2 (GLT-1; slc1a2); EAAT3, excitatory amino acid carrier (EAAC1; slc1a1); EDTA, sodium ethylenediamine tetraacetate; GABA, gamma-aminobutyric acid; GAT1, GABA transporter 1 (slc6a1); GAT2, GABA transporter 2 (slc6a13); GAT3, GABA transporter 3 (slc6a11; mGAT4); KO, knockout; NaPi, sodium phosphate buffer with pH 7.4; SDS, sodium dodecyl sulfate; VGLUTs, vesicular glutamate transporters; WT, wild-type mice.

Abstract

Immunocytochemistry and Western blotting are still major methods for protein localization, but they rely on the specificity of the antibodies. Validation of antibody specificity remains challenging mostly because ideal negative controls are often unavailable. Further, immunochemical labeling patterns are also influenced by a number of other factors such as post mortem changes, fixation procedures and blocking agents as well as the general assay conditions (e.g. buffers, temperature etc). Western blotting similarly depends on tissue collection and sample preparation as well as the electrophoretic separation, transfer to blotting membranes and the immunochemical probing of immobilized molecules. Publication of inaccurate information on protein distribution has downstream consequences for other researchers because the interpretation of physiological and pharmacological observations depends on information on where ion channels, receptors, enzymes or transporters are located. Despite numerous reports, some of which are strongly worded, erroneous localization data are being published. Here we describe the extent of the problem and illustrate the nature of the pitfalls with examples from studies of neurotransmitter transporters. We explain the importance of supplementing immunochemical observations with other measurements (e.g. mRNA levels and distribution, protein activity, mass spectrometry, electrophysiological recordings, etc) and why quantitative considerations are integral parts of the quality control. Further, we propose a practical strategy for researchers who plan to embark on a localization study. We also share our thoughts about guidelines for quality control.

Introduction

An interest in utilizing antibodies as analytical tools began in the 1930s when investigators started to conjugate antibodies with other molecules. Albert H. Coons showed in the early 1940s that it was possible to use fluorescently tagged antibodies to localize antigens in tissue sections (Coons and Kaplan, 1950; Coons, 1971). Since then a multitude of different immunochemical methods have been developed. Western (protein) blotting was invented (Towbin *et al.*, 1979) after the discovery that sodium dodecyl sulfate (SDS) could enhance the resolving power of electrophoresis gels (Laemmli, 1970). The methods became popular, but along with enthusiasm came concerns about the validity of the data generated (Petrusz *et al.*, 1976; Petrusz *et al.*, 1980; Petrusz, 1983). Some investigators even questioned if specificity could ever be proven (Swaab *et al.*, 1977). Nevertheless, the techniques have been in widespread use for several decades for identifying individual proteins in complex biological samples (e.g. tissue extracts and sections), and have resulted in an enormous amount of new knowledge.

However, despite the understanding of the principles, the field of immunocytochemistry is still troubled by spurious results due to insufficient controls for specificity (e.g. Pool and Buijs, 1988; Griffiths, 1993; Brandtzaeg, 1998; Grube, 2004; Ramos-Vara and Miller, 2014; Holmseth *et al.*, 2012a; Griffiths and Lucocq, 2014).

In the present review, we try to explain the difficulties and still convey a positive message: how to do localization well. Although there are arguments in favor of detailed formal guidelines, it is hard to define the exact tests that should be performed (e.g. Saper and Sawchenko, 2003; Saper, 2005; Holmseth *et al.*, 2006; Rhodes and Trimmer, 2006; Lorincz and Nusser, 2008; Fritschy, 2008; Saper, 2009;

Burry, 2011; Griffiths and Lucocq, 2014). After all, each project is unique and that uniqueness comprises both unique challenges and unique opportunities. Instead of formal rules we suggest a practical strategy which we illustrate with examples mostly from studies of neurotransmitter transporters in the brain (Zhou and Danbolt, 2013). This review has, however, broader implications as the localization of transporters is used to exemplify the trickier aspects of antigen-antibody interactions: the same rationale can be applied to almost any biomarker detection in cells and in tissues sections.

The extent of the problem is such that it should be taken seriously

Several recent reports document poor specificity of commercially available antibodies. For instance, seven papers appeared in Naunyn-Schmiedebergs Archives of Pharmacology in 2009 highlighting a lack of selectivity of 49 antibodies against 19 subtypes of alpha(1)- and beta-adrenoceptors, muscarinic, dopamine and galanin receptors as well as vanilloid (TRPV1) receptors. These data demonstrate that lack of selectivity appears to be the rule rather than the exception for antibodies against G-protein-coupled and perhaps also other receptors (Michel *et al.*, 2009; Kirkpatrick, 2009). Indeed, there are several reports of poor commercial antibodies to adrenergic receptors (Hamdani and van der Velden, 2009; Jensen *et al.*, 2009) as well as to other proteins such as acetylcholine receptors (Herber *et al.*, 2004; Moser *et al.*, 2007), histones (Egelhofer *et al.*, 2011), CD95 ligand (Strater *et al.*, 2001), angiotensin receptors (Hafko *et al.*, 2013; Herrera *et al.*, 2013; Elliott *et al.*, 2013), dopamine receptors (Bodei *et al.*, 2009), cannabinoid receptors (Snyder *et al.*, 2010; Ashton, 2012; Morozov *et al.*, 2013), histamine receptors (Beermann *et al.*, 2012), P2X receptors (Ashour *et al.*, 2006) and others.

Together, these examples describe many poorly specific antibody products to about 100 different proteins. Researchers have been using precious research funding to buy these products, and subsequently waste even more resources when they use them to generate data. Fortunately, most of these data do not get published, but some do. And that may mislead other researchers to do less interesting investigations than they otherwise could have done. Even worse, some of these antibodies are used in medical diagnostics with potential consequences for patients.

One problem is poor testing when a product is first put on sale. Another is batch differences. Thus, when a researcher purchases a product with the same

product number, the properties of the antibodies may be significantly different. This has also been pointed out by multiple investigators (e.g. Strater *et al.*, 2001; Bodei *et al.*, 2009; Jensen *et al.*, 2009; Herber *et al.*, 2004; Grimsey *et al.*, 2008; Kirkpatrick, 2009; Herrera *et al.*, 2013; Bohmer *et al.*, 2014; Baker, 2015; Van Liefferinge *et al.*, 2016).

We therefore fully agree with those who argue that commercial antibody producers should test their antibodies more rigorously before selling them to scientists or pathologists who often lack the resources or expertise to evaluate acquired antibodies (Rhodes and Trimmer, 2006; Pradidarcheep *et al.*, 2008; Couchman, 2009; Boenisch, 2006; Kalyuzhny, 2009; Bohmer *et al.*, 2014; Baker, 2015).

However, not all testing can be done in advance because the overall labeling specificity is affected by so many parameters that antibodies have to be tested for each application (e.g. Ottersen, 1987; Holmseth *et al.*, 2006; Lorincz and Nusser, 2008; Rhodes and Trimmer, 2006). Virtually all assay conditions can affect antibody binding, including protein conformation and hydrophobic interactions (e.g. pH, buffer composition, ionic strength), tissue handling steps (e.g. time to fixation, type of fixation, fixative composition, fixation time, storage after fixation) and antigen retrieval techniques.

So although the antibody producers do deserve criticism as explained above, neither the researchers themselves (Roth, 2006; Rhodes and Trimmer, 2006; Schonbrunn, 2014; Lorincz and Nusser, 2008) nor the editors of journals should avoid their responsibility (Smith, 2006).

Unfortunately, the problem is increasing due to highly sensitive imaging techniques, the ease by which images can be acquired and the pressure to publish.

Immunocytochemistry is a method that is able to produce publishable, but potentially incorrect data, at a high rate and low cost (Rhodes and Trimmer, 2006) This is a major concern considering the widespread use of these methods, the considerable effort required to correct inaccurate results and the downstream consequences for other researchers (e.g. Morrow and Friedrich, 2003; Roth, 2006; Rhodes and Trimmer, 2006; Steingart *et al.*, 2007; Smith, 2006; Fritschy, 2008; Couchman, 2009; Kalyuzhny, 2009; Holmseth *et al.*, 2012a; Herrera *et al.*, 2013; Griffiths and Lucocq, 2014). After all, the biomedical research community relies directly or indirectly on precise protein localization data because interpretation of the other methods (e.g. electrophysiological and pharmacological observations) depends on information on where ion channels, receptors, enzymes or transporters are located.

[Figure 1 about here]

Why it is difficult to verify labeling specificity

A good antibody binds to the desired target with high affinity, allowing it to be used at concentrations well below the concentration where it starts to bind to other targets (see Fig. 1). The challenge, however, is that the number of possible antibody binding sites (epitopes) in a tissue section is virtually infinite, and their affinities for a given antibody are unknown. Consequently, it is hard to rule out the existence of unknown epitopes with high affinity for the antibody.

Obviously, antibodies are protein molecules which recognize the antigens much like receptor proteins recognize ligands or enzymes recognize substrates (Pool and Buijs, 1988; Griffiths, 1993; Holmseth *et al.*, 2005). It should be recalled how medicinal chemists manage to develop new molecules that can compete with endogenous ligands despite very different chemical structures. From this perspective

it is not surprising that antibodies may cross-react with seemingly unrelated molecules. Since we made the first antibodies to a glutamate transporter (Danbolt *et al.*, 1992), our laboratory has produced about one thousand different antibodies. Even after affinity purification, most still displayed some degree of cross-reactivity with unrelated molecules (Holmseth *et al.*, 2012a). Thus, cross-reactivity is common (Davies *et al.*, 2007) although the identities of the cross reacting molecular species are rarely determined.

Cross-reactivity does not have to imply that an antibody preparation is contaminated with unwanted antibodies derived from other B-cell clones. It can be due to the same antibodies as those recognizing the antigen under study (Danbolt *et al.*, 1998; Holmseth *et al.*, 2005; Wilson *et al.*, 1996). Consequently, even monoclonal antibodies can display cross-reactivity. In fact, when we made monoclonal antibodies to EAAT2 (Levy *et al.*, 1993) we also isolated clones producing polyreactive antibodies (Danbolt *et al.*, 1998). The cross-reactivity is often unexpected. For instance, antibodies to a glutamate transporter (Holmseth *et al.*, 2005) and carbonic anhydrase (Li *et al.*, 2009) cross-reacted with tubulin. Similarly, anti-DNA antibodies recognized peptide sequences (Sibille *et al.*, 1997; James *et al.*, 1999), dextran sulfate (Kinoshita *et al.*, 1989) and even the NR2 glutamate receptor (DeGiorgio *et al.*, 2001). Mitochondria represent a frequent site of cross-reactivity (e.g. Holmseth *et al.*, 2006; Yang *et al.*, 2006; Morozov *et al.*, 2013). Reactivity with unrelated epitopes is elegantly illustrated when phage display is used to test antibodies (Sibille *et al.*, 1997; Menendez and Scott, 2005).

It should also be realized that tissue processing and fixation chemically modifies the tissue (Rasmussen and Albrechtsen, 1974; Somogyi and Takagi, 1982; Berod *et al.*, 1981; Leong and Gilham, 1989; Korogod *et al.*, 2015), leading to the

creation and elimination of epitopes. For instance, a monoclonal antibody to vimentin reacted with enamel proteins, but only after glutaraldehyde fixation (e.g. Josephsen *et al.*, 1999; Willingham, 1999). Fixation of immunoblots prior to immunolabeling is a simple inexpensive way to get some assessment of the effect of this manipulation (Holmseth *et al.*, 2006; Holmseth *et al.*, 2012a).

Reporter mice as an independent verification

A large number of genetically modified mouse lines (Table 1) are now available to the public (e.g. Heintz, 2001; Nagy and Mar, 2001; Mori *et al.*, 2006; Pfrieger and Slezak, 2012). The genetic modifications not only comprise deletion of genes (knockout mice), but also insertion of DNA that was not there originally. The inserted DNA can for instance be encoding enzymes or fluorescent proteins (e.g. Livet *et al.*, 2007). Reporter mice, where promotor activation results in expression of fluorescent proteins, can be used as an alternative, or as a supplement, to immunocytochemistry (Nolte *et al.*, 2001). This offers new opportunities for studies of cell progeny (e.g. Malatesta *et al.*, 2003; Zhu *et al.*, 2008; Platel *et al.*, 2009; Huang *et al.*, 2014), visualization of astrocytes and astrogliosis in living brain tissue (Nolte *et al.*, 2001; Weimer *et al.*, 2008; Tang *et al.*, 2009) and other dynamic interactions which are hard to study immunocytochemically (e.g. Reichenbach *et al.*, 2010; Young *et al.*, 2010; Herzog *et al.*, 2011). There are, however, issues with reporter mice also as the transcriptional activity can be influenced by multiple factors. Therefore the expression patterns can be altered depending on how promotor elements are affected (Yeo *et al.*, 2013). Nevertheless, the distribution of fluorescence in reporter lines for the EAAT1, EAAT2 and EAAT4 glutamate transporters (Regan *et al.*, 2007; Gincel *et al.*, 2007; de Vivo *et al.*, 2010a; de Vivo *et al.*, 2010b) is in good agreement with previous

immunocytochemistry (Lehre *et al.*, 1995; Dehnes *et al.*, 1998; Zhou and Danbolt, 2014). A review of the potential of genetically modified animals, however, is beyond the scope of this review.

The expression levels required for physiologically significant function

How much protein is necessary to account for the proposed or measured function? This question becomes more important as increasingly sensitive detection techniques are developed. In this context, it is worth noting that most DNA is transcribed (Birney *et al.*, 2007). Although it is not known if all mRNA is translated, it should be asked whether the detected molecules have the capacity to accomplish the proposed or measured tasks at physiologically relevant rates. The number of molecules needed to accomplish a given task depends on what that task is. Some proteins (e.g. primary activators of a cascade system such as the complement system) can deliver significant effects when present in minute quantities, while other proteins may only make a difference if highly expressed.

Neurotransmitter transporters are examples of proteins that need to be present in high numbers because co-transport is a relatively slow process requiring tens of milliseconds for completion of a single transport cycle (e.g. Grewer and Rauen, 2005; Karakossian *et al.*, 2005; Gonzales *et al.*, 2007; Gameiro *et al.*, 2011; Zhou *et al.*, 2014a; Hanson *et al.*, 2015). Thus, one transporter molecule can only transport a couple of dozen substrate molecules per second at Vmax. Sub-millisecond transmitter removal requires more vacant binding sites (transporter molecules) than released neurotransmitter molecules. This is because low molecular mass compounds, such as amino acid neurotransmitters, diffuse quickly out of the synaptic cleft on a low microsecond timescale until they bind to transporters and are

removed (e.g. Clements, 1996; Tzingounis and Wadiche, 2007). Another reason why high numbers of transporters are required follows from the low resting levels of extracellular GABA (e.g. Westergren *et al.*, 1994) and glutamate (Herman and Jahr, 2007) despite rapid neurotransmitter release (e.g. Jabaudon *et al.*, 1999). Maintenance of resting levels far below the Km-values of the transporters (Danbolt, 2001; Conti *et al.*, 2004) requires a vast excess of transporters (Bergles and Jahr, 1997; Otis and Kavanaugh, 2000; Herman and Jahr, 2007) in agreement with biochemical measurements of transporter concentrations (Lehre and Danbolt, 1998; Holmseth *et al.*, 2012b).

In contrast to fast transmitter removal, less demanding needs that can be satisfied in minutes or hours rather than milliseconds may require fewer transporters. Thus, if a physiological role can be demonstrated and the numbers of protein molecules are insufficient, then it should be asked whether the protein can mediate its effect via novel mechanisms that require fewer molecules e.g. by acting as a receptor or ion channel. For instance, tens of thousands of ions may pass through an ion channel per second implying that relatively few channels can mediate significant ion fluxes. In fact, the EAAT4 (slc1a6) and the EAAT5 (slc1a7) glutamate transporters may function as glutamate gated anion channels (Dehnes *et al.*, 1998; Veruki *et al.*, 2006; Gameiro *et al.*, 2011; Schneider *et al.*, 2014).

Another parameter to consider is if the cells supposed to be expressing a given transporter can make use of it, e.g. do they have enough energy to operate all of the transporters, and is their plasma membrane surface area large enough to accommodate all of the transporter molecules? This argument is particularly relevant when reporting expression of transporter proteins in endothelial cells which are flat with few mitochondria and fairly smooth plasma membranes. The opposite are cells

like those found in the proximal tubules in the kidneys that are packed with mitochondria and have huge surface areas due to abundant microvilli. Consequently, a large number of transporter molecules can fit into their plasma membranes, and the cells have energy to fuel them.

Suggested strategy when embarking on a localization project

1. Does the tissue of interest perform functions attributable to the protein to be localized?

Before embarking on a project, it is a good idea to verify that the tissues of interest actually express the antigen to be studied at levels that may be functionally relevant. Can protein function be measured (e.g. transport activity or enzymatic activity)? Will these protein levels be detectable? Are other proteins with similar function expressed in the same tissue at higher concentrations? Glutamine transporters illustrate this point as there are at least 14 of them (Bhutia and Ganapathy, 2015). It may be worthwhile to search available microarray, transcriptome and proteome datasets (e.g. Lu *et al.*, 2009; Walther and Mann, 2011; Ulrich *et al.*, 2014; Holtman *et al.*, 2015) as these may give good indications of which proteins it may be possible to detect.

2. Sample quality - proteolysis and true oligomers versus in vitro aggregation

Since the tissue samples to be studied represent the material on which the entire study is founded, both the quality and the processing of the samples are key factors.

Several general descriptions are available on how to preserve protein stability (e.g. Deutscher, 1990), how to purify (e.g. Linn, 1990), how to isolate membranes and make extracts (e.g. Dignam, 1990; Ozols, 1990) and how to solubilize (e.g. Neugebauer, 1990; Hjelmeland, 1990). In fact, multitudinous methods have been

developed for different purposes, but this is beyond the scope of this review. Here we only want to point out two factors that have caused some confusion within studies of glutamate transporters, namely (a) post-mortem proteolysis and (b) differences between true oligomers and cross-linking resulting from sample preparation.

(a) Post-mortem artifacts (Figs. 2 and 3): Tissue from autopsies has proven invaluable in studies of diseases, including neurological diseases, but the post-mortem interval (time from death to tissue preservation) can influence the results of the investigations. Changes are readily detected by proteomic analyses after 6 hours (Machaalani *et al.*, 2010). If all proteins had been degraded at the same rate in all cells, then the post-mortem interval would only have affected the sensitivity. However, this is not the case. The rates of degradation vary greatly between different proteins and between brain regions (Patel *et al.*, 1993; Martin *et al.*, 2003; Wang *et al.*, 2000; Li *et al.*, 2012; Rutkiewicz and Basu, 2012). This might in fact be expected considering that the distributions of proteins (enzymes included) are not uniform, and that many of the post-mortem alterations result from dynamic processes (Geddes *et al.*, 1995; Goni-Oliver *et al.*, 2009; Yeh *et al.*, 2009; Li *et al.*, 2012). For instance, the N- and C-termini of EAAT2 glutamate transporter (GLT-1; slc1a2) degrade faster than those of its close relative, EAAT1 (GLAST; slc1a3) which in turn degrade faster than the C-terminus of EAAT3 (EAAC1; slc1a1). In contrast, epitopes within central parts of the EAAT2 protein, e.g. residues 107-120 and 493-508 (Beckstrøm *et al.*, 1999; Li *et al.*, 2012) as well as to residues 146-161 (Fig. 2) are far more resistant. Similarly, the NR2A and NR2B glutamate receptor subunits are proteolyzed faster than the other NMDA and AMPA types of glutamate receptors (Wang *et al.*, 2000). Further, EAAT2 is proteolyzed faster in the cerebral cortex than in the striatum (Li *et*

al., 2012).Thus, the labeling pattern obtained depends both on the post mortem interval and the antibodies used (Tessler *et al.*, 1999; Li *et al.*, 2012).

[Figure 2 about here]

[Figure 3 about here]

(b) *True oligomers vs artifactual cross-linking*: Another factor that has been confusing to researchers studying glutamate transporters is preservation of *in vivo* oligomeric structure versus *in vitro* cross-linking or aggregation (Figs. 4 and 5). The EAAT2 glutamate transporter exists in the brain as homo-trimers where the subunits are non-covalently attached to each other (Haugeto *et al.*, 1996; Yernool *et al.*, 2004; Gendreau *et al.*, 2004). When fresh brain tissue is rapidly homogenized directly in sodium dodecyl sulfate (SDS), only monomers are seen on the Western blots regardless of whether reducing agents have been added (Fig. 4, Lane 1). The native oligomers can be visualized if they are preserved by chemical cross-linking prior to solubilization of the membranes in which they reside (see Fig. 5A).

If the intact brain membranes are oxidized (Fig. 4, Lane 2), then covalent bonds form between one EAAT2 subunit and other molecules (Trotti *et al.*, 1998; Danbolt, 2001). Whether this is another EAAT2 subunit, or something else, is not known at the moment. These complexes, however, are not seen if the samples are subjected to reducing agents or if oxidation occurs after solubilization in SDS.

[Figure 4 about here]

The phenomenon that causes confusion is therefore a different one: the solubilization procedure affects the electrophoretic mobility pattern of EAAT2. If the tissue is solubilized with mild detergents (e.g. cholate or Triton X-100) and not in SDS, then complexes form even under reducing conditions (Fig. 5B). Once formed, these complexes are resistant to SDS. The formation is enhanced at elevated temperatures

(Fig. 5B) and prevented by addition of phospholipids (not shown). To what extent this reflects irreversible attachment of subunits in endogenous oligomeric complexes or later aggregation *in vitro* depends on the *in vitro* conditions (e.g. protein and salt concentrations). Thus, it is important to realize that variations in sample handling have major effects and that it should not be taken for granted that changes in the proportions between monomers and oligomers observed on Western blots reflect real differences in oligomeric structure *in vivo*. Unfortunately, this assumption is now commonly found in the literature. For instance, a number of investigators have used the so called "RIPA Lysis and Extraction Buffer" which contains SDS in combination with deoxycholate and sometimes a non-ionic detergent. When brain tissue is solubilized in this buffer, oligomeric bands are seen. This buffer is not suitable for quantitative examination of glutamate transporters.

[Figure 5 about here]

A related point that merits emphasis is the difficulty in quantifying proteins on Western blots when the proteins are distributed between monomers and dimers or higher order aggregates. The reason is that the labeling intensity measured depends on several factors such as the efficiency of solubilization of the protein under study, the entry into the electrophoresis gel, the percentage that leaves the gel during electrophoretic transfer, the percentage that is captured on the blotting membrane and the availability of the epitopes for antibody binding. One or more of these parameters are likely to differ between monomers and multimers.

3. Acquisition of antibodies

When obtaining antibodies from others, it is important to make sure that all relevant documentation is available. We fully agree that data obtained with insufficiently

documented antibodies, should not be published (e.g. Saper and Sawchenko, 2003; Saper, 2005; Rhodes and Trimmer, 2006; Fritschy, 2008; Holmseth *et al.*, 2012a). Both the source of the antibody and the exact antigen used for immunization should be known to the investigators, at least for main antibodies that a study relies on. If the antiserum was purified in some way, then this should also be recorded. As explained above, there may be significant differences between antibody batches so it is important to refer to the exact batch (e.g. batch number, production date). When monoclonal antibodies are produced in the form of ascites fluid, then they will be contaminated with other antibodies from the host mouse. These contaminating antibodies may vary from batch to batch. The batch number should therefore be recorded also when monoclonal antibodies are used.

Obviously, it is an advantage if several different antibodies to the same target can be obtained. Then it can be seen whether they all give the same results.

If the plan is to do extensive studies of a particular protein, it is worthwhile to make the antibodies rather than buying them. That is costly, but if large amounts are needed, then it may still be cheaper and safer than purchasing and testing several small aliquots from different batches from commercial suppliers. Antibody production is straight forward. We typically immunize with synthetic peptides and prefer a "shotgun-approach": we select the hydrophilic portions of the termini (the longer the better) as well as other parts of the protein, mix the peptides together with carrier protein and glutaraldehyde, and inject this subcutaneously (for details see: Danbolt *et al.*, 1998; Holmseth *et al.*, 2005). We avoid intracutaneous injections as they cause unnecessary suffering.

4. What is the evidence that the antibody recognizes the antigen?

Unfortunately, it cannot even be taken for granted that an antibody binds to the antigen at all. Therefore, one of the first things to do is to obtain a positive control: a sample that contains the native antigen of interest in high concentrations. For instance, mature forebrain tissue is a very good source of EAAT1, EAAT2, GAT1 and GAT3 (Lehre *et al.*, 1995; Ribak *et al.*, 1996; Conti *et al.*, 2004; Melone *et al.*, 2015), while cerebellum and kidney are, respectively, good sources of EAAT4 (Dehnes *et al.*, 1998) and EAAT3 (Holmseth *et al.*, 2012b). Transfected cell cultures may be chosen for proteins of unknown distribution or for proteins only expressed at low levels.

It is important that the antigen used as a positive control resembles the natural one as much as possible. For instance, immunization with peptides usually gives antibodies that recognize the peptides, but many of these antibodies (the majority in fact) do not recognize the intact protein for various reasons (see: Danbolt *et al.*, 1998). The choice of method for testing depends on how the antibodies have been made. Immunoblotting is excellent for most antibodies to synthetic peptides or purified proteins, but is not optimal in cases where the antibodies have been produced and selected for their ability to recognize protein complexes or specific conformations. However, conformation specific antibodies are exceptions so positive proof is required before arguments that antibodies work for immunocytochemistry and not for immunoblotting can be accepted. It is also worth checking that the antibodies recognize the antigen after the same treatment as the tissue will be subjected to (fixation, embedding, antigen retrieval etc: Josephsen *et al.*, 1999; Holmseth *et al.*, 2006; Holmseth *et al.*, 2012a). This will also give a first indication of the sensitivity that can be obtained.

5. Specificity testing by immunoblotting

As stated above, most antibodies should be tested by immunoblotting. It is important to make extracts from the tissues in which the distribution of the antigen is going to be studied because the gene expression profiles vary greatly between cell types. It is essential to test the antibodies on blots containing as many tissue antigens as possible, so whole tissue homogenates should be used. However, non-transporter molecules present in the samples may block binding of the transporters to the membranes (Fig. 6). This was noted a long time ago (Danbolt *et al.*, 1992) and can, at least partly, be avoided by homogenizing the tissue in water and centrifuging to separate water soluble and water insoluble proteins. In fact, when we made antibodies to the betaine-GABA transporter (BGT1; slc6a12) we initially thought that the antibodies did not work and we made new antibodies. About ten years later, we homogenized the transfected cells in water to remove the water soluble proteins. Then we realized that many of the antibodies were excellent, but that BGT1 had not been immobilized on the blotting membranes due to interference from non-BGT1 proteins (Zhou *et al.*, 2012a).

[Figure 6 about here]

Separation of water soluble and water insoluble proteins can be used to test specificity. Because transporter proteins are integral membrane proteins, they are supposed to be found in the membrane fraction. If the immunoreactivity is in the water soluble fraction, this is a warning that the antibodies bind to something other than an integral membrane protein. Extracts from mouse pancreas illustrate this (Fig. 7). EAAT2 antibodies cross-react with a water soluble protein with a molecular mass that is similar to that of EAAT2. This cross-reactive protein is present in both wildtype mice and EAAT2 knockout mice. EAAT2 protein itself is not detectable in young adult

mouse pancreas (Zhou *et al.*, 2014b). The identity of the cross-reacting antigens is unknown, but it is interesting that islet cells contain several proteins that often give rise to autoantibodies (Arvan *et al.*, 2012). Some of these proteins have molecular masses similar to that of EAAT2, and such antibodies may be present in rabbit sera.

Contaminating cross-reactive antibodies often require active removal by absorption. This is illustrated with antibodies to a peptide representing residues 479-492 of rat EAAT3 (Bjørås *et al.*, 1996). This peptide gave rise to antibodies with high affinity to both tubulin and EAAT3 (Holmseth *et al.*, 2005). The antisera had first to be passed through a column with immobilized tubulin to remove antibodies reacting with tubulin. Then the remaining antibodies with affinity to EAAT3 could be isolated.

[Figure 7 about here]

In our experience, immunoblots are informative: antibodies that look specific on blots are often specific in sections. Nevertheless, exceptions are common so immunoblots should be supplemented with other tests whenever possible (Holmseth *et al.*, 2005; Holmseth *et al.*, 2012a). Further, cross-reactivity can be highly specific and localized (Josephsen *et al.*, 1999; Holmseth *et al.*, 2012a). It is not surprising that antibodies may display different degrees of specificity when tested on immunoblots and on sections considering that the former is based on molecules that have been solubilized. The molecules may have different conformations and are likely to be separated from their natural molecular neighbors during electrophoresis. Further, the smallest and the largest molecules are lost, and the three dimensional structure of the tissue is destroyed. In contrast, the three dimensional structure is preserved in sections, but the tissue is often chemically modified and some components may be lost depending on the tissue processing used. It is also important to remember that

one band on an immunoblot may contain more than one protein (Holmseth *et al.*, 2012a).

6. Testing on tissue sections

If a signal cannot be obtained on tissue sections, then the technique has to be modified. However, as illustrated (Fig. 1), a signal can usually be obtained by adjusting fixation conditions, blocking conditions, salt concentrations, pH and antibody concentrations or by employing antigen retrieval techniques (e.g. Shi *et al.*, 2011) and methods for labeling enhancement such as gold-silver intensification (e.g. Dobo *et al.*, 2011) and tyramide signal amplification (Kerstens *et al.*, 1995; Silahtaroglu *et al.*, 2007). It is therefore necessary to ask critical questions such as: does this labeling only represent the antigen of interest or is it due to something else instead of, or in addition to, the antigen of interest? Immunolabeling does not in itself prove that the protein is present. Beware of sampling error: "*Finally I found an antibody that worked!*" What is the definition of a good antibody? Unfortunately, quite often a good antibody is one that gives the expected or desired labeling. This brings up another factor, namely quality control by an experienced scientist and proper training of new recruits. Thus, sampling errors like this may occur if the communication between a junior team member and the principal investigator is insufficient.

If the labeling represents the right antigen, then the tissue distribution in sections should match the labeling of the corresponding bands on immunoblots. A simple test is to dissect regions with different labeling intensities and then check if the labeling intensities obtained on immunoblots match those seen in tissue sections. Discrepancies should raise concerns about antibody specificity. However, differences

in tissue water content may affect fixation and thereby antibody penetration into the tissue (see discussion about the use of detergents in: Danbolt *et al.*, 1998; Heffer-Lauc *et al.*, 2007). Also as explained above, (Fig. 5), non-transporter proteins may interfere with the binding of transporters to the blotting membranes causing underestimation of expression levels.

The strength of these tests will be greater if several antibodies give the same result. However, variable splicing can be a reason they may not. And if they give the same result, then it is still possible that they all have the same cross-reactivity as illustrated above with pancreas (Fig. 6) and shown by others (Davies *et al.*, 2007). Further, if the antibody concentration needed for obtaining labeling of sections is considerably higher than that needed to label Western blots, then this may be due to the fundamental differences between blots and sections (see above). Alternatively, it might be a sign that the data do not quite fit with the working hypothesis.

7. Testing on genetically modified tissue

If the antibodies look promising, it is worthwhile to find out if suitable genetically modified organisms exist. Knockout animals are animals where a gene has been deleted and represent very powerful negative controls (e.g. Herber *et al.*, 2004; Holmseth *et al.*, 2005; Holmseth *et al.*, 2012a; Li *et al.*, 2013; Cecyre *et al.*, 2014; Van Liefferinge *et al.*, 2016; Baek *et al.*, 2013; Bohmer *et al.*, 2014). The main problem is their availability and the fact that most are mice while most immunocytochemistry is done on rat and human tissue. However, with human samples in particular, there may not be any good negative controls at all. So despite obvious limitations, tissue from knockout mice may still be the best negative control available. Fortunately, a huge number of animals with various modifications of their

genomes are now available and the list is rapidly becoming longer (e.g. Nagy and Mar, 2001; Skarnes *et al.*, 2011; Table 1). For instance, knockout mice for most of the glutamate and GABA transporter genes are available: EAAT1 (Watase *et al.*, 1998), EAAT2 (Tanaka *et al.*, 1997; Zhou *et al.*, 2014b), EAAT3 (Peghini *et al.*, 1997), EAAT4 (Huang *et al.*, 2004), glutamate-cystine exchanger (xCT; slc7a11; Sato *et al.*, 2005), GAT1 (Chiu *et al.*, 2005), GAT2 (Zhou *et al.*, 2012b) and BGT1 (Lehre *et al.*, 2011; Zhou *et al.*, 2012a). In conventional (global) knockout animals the target gene is absent already before conception and is therefore absent in all cells during development. One advantage for use as negative controls in immunocytochemistry experiments is that all cells lack the gene. On the other hand no mice will be born if the deletion is lethal early in development. Although compensatory changes or downstream consequences of the deletion may complicate interpretation, such animals have nevertheless been extremely useful.

The EAAT2 knockout mice (Tanaka *et al.*, 1997) illustrate several points. They are inconspicuous at birth because EAAT2 is hardly expressed (Ullensvang *et al.*, 1997), but become hyperactive and develop epilepsy after three weeks. About half of them die suddenly before the end of the fourth week. This agrees with biochemical studies showing that EAAT2 is the major glutamate transporter in adult brain (Danbolt *et al.*, 1992; Otis and Kavanaugh, 2000).

Another limitation of knockout animals can be other genes containing the same sequence, or residual expression of the deleted gene. It is common to select a few critical exons that are necessary for function. Although this eliminates the function, a truncated protein may still be expressed unless the DNA is deleted in such a way that a frame-shift is introduced resulting in both a stop codon and a

meaningless protein sequence. Unfortunately, not all knockouts have been constructed with this in mind.

Further, when a gene is deleted, this may affect expression of other genes (Teng *et al.*, 2013; Ghule *et al.*, 2015). Cross-reactive molecules may be down-regulated or up-regulated. However, if an antibody gives rise to labeling in knockout tissue, then this should not be taken lightly. Unless proven otherwise, labeling of samples from knockout animals should be considered cross-reactivity. Obviously, this argument is only valid if the protein is truly absent in the knockout animals as explained above.

It is also important that the conditions used during specificity testing have to match the conditions during data acquisition (e.g: Lorincz and Nusser, 2008; Rhodes and Trimmer, 2006). The optimal solution is to process tissue from wild-type and knockout littermates together. This may not always be possible, but the testing should at least be done with the same method. Further antibody specificity tests should be conducted in the specific tissue to be examined, as the cross-reactivity can be regional or tissue-specific (Everaerts *et al.*, 2009; Holmseth *et al.*, 2012a; Ashour *et al.*, 2006).

Comment on the pre-adsorption control

Antigen pre-adsorption was originally introduced to validate antisera containing mixtures of antibodies with a large variety of specificities (e.g. Pool and Buijs, 1988). This test tells if the labeling is due to the same antibodies as those recognizing the antigen. Importantly, it does not tell if the observed labeling represents a specific visualization of the antigen under study or if it is due to cross-reaction with other molecules (e.g. Swaab *et al.*, 1977; Pool and Buijs, 1988; Burry, 2000). Despite this,

pre-adsorption is still regarded by many as an obligate control for the verification of immunocytochemical labeling - even labeling obtained with monoclonal and affinity purified antibodies. This is unfortunate as the pre-adsorption test can give a misleading impression of specificity (for illustrations see: Holmseth *et al.*, 2012a). Compounding this problem, it is often costly to obtain enough free antigen to perform the test, diverting time and resources from more definitive experiments.

Correlation between labeling intensity in tissue sections and protein levels

As noted above, quantitative considerations are important, not only to understand function, but also to judge specificity. Are the expression levels high enough to be detectable? If not, then the labeling may be artifactual.

The high expression levels of glutamate transporters (Lehre and Danbolt, 1998) are part of the reason why the first post-embedding immunogold electron micrographs of EAAT1, EAAT2 and EAAT4 (Chaudhry *et al.*, 1995; Dehnes *et al.*, 1998) were so successful: *there were sufficient numbers of EAAT molecules in the plane of the section to give convincing labeling*. This also explains, at least partly, why we initially failed to detect EAAT2 in axon-terminals. Despite early reports suggesting glutamate uptake by nerve terminals, it took a long time to realize that this was due to EAAT2 (Danbolt *et al.*, 2016).

The reasons why post-embedding immunogold electron microscopy (van den Pol, 1989; Ottersen, 1989) has relatively low sensitivity is that only the proteins that are in the exact sectioning plane are detected (for method see: Danbolt *et al.*, 1998; Amiry-Moghaddam and Ottersen, 2013). The tissue sections used for electron microscopy are thin (45-90 nm) and not much thicker than the outer diameter of synaptic vesicles (40 nm), and only a few times thicker than the width of the neuronal

synaptic cleft (usually 20 nm). Further, despite the thinness, the antibodies do not penetrate well into the sections because of the resins in which the tissue is embedded. To maximize labeling intensity, sections are often labeled on both sides. Thus, the antibodies label only the molecules exposed at the surface. Chemical modification (fixation etc) can reduce the availability of epitopes even more. A high density of gold particles along a cut membrane is therefore only expected if the expression levels are very high. The sensitivity of post-embedding is thus low compared to e.g. fluorescent based labeling of free-floating sections. Consequently, if labeling is seen with immuno-gold and not with fluorescence based microscopy, then there is a mismatch which probably warrants extra control experiments.

Another challenge follows from the vulnerability of the ultrathin sections and thereby also the labeling. These sections are easily damaged during processing. Parts of the sections may be missing and the labeling uneven on the remaining parts. Consequently, there is variability and this leads to another challenge: enforcing strict quality controls in image acquisition to avoid sampling error. This challenge comes in addition to those mentioned above (specificity, proteolysis etc) and represents another example of a situation where results may depend on experience. Students may be inspired by an exciting hypothesis and go to the microscope to photograph unconsciously what the principal investigator hypothesized. A number of images are acquired and subjected to statistical analyses. The principal investigator may not realize that image collection is already biased.

To understand brain tissue, it is important to relate to the sizes of the cellular extensions. The following calculation may serve as an illustration: There is about one synapse per cubic micrometer brain tissue (gray matter; for references see: Danbolt, 2001), and there are 10^{15} cubic micrometers in one liter. If this number is divided by

Avogadros number, it follows that the concentration of synapses is in the equivalent of approximately 1 nanomol/liter. The purpose of this rather unorthodox way of expressing synapse density is to illustrate that a protein has to be highly expressed if it is present in several copies per glutamatergic synapse (which represents the majority of brain synapses).

The sizes of the tiny cellular extensions making up brain tissue (the neuropil part of it) may be easier to grasp if they are compared to a red blood cell (erythrocyte). A human erythrocyte is about 7 μm in diameter. This means that the diameter of a red blood cell is about 9 times larger than the scale bar shown in figure 8. A plasma membrane is about 5 nm thick and the width of the extracellular space (the distances between neighboring cellular extensions) is typically in the range 20-40 nm, while the diameter of a glutamate transporter trimer is believed to be about 8 nm (Yernool *et al.*, 2004). Cellular elements are tightly intermingled (Kirov *et al.*, 1999; Sorra and Harris, 2000; Witcher *et al.*, 2010; Harris and Weinberg, 2012; Mathiisen *et al.*, 2010). This is schematically illustrated in figure 8. This means that the total amount of plasma membrane is large: about 14 $\mu m^2/\mu m^3$ in the stratum radiatum, rat hippocampus CA1 (Lehre and Danbolt, 1998).

[Figure 8 about here]

The closeness of neuronal and glial membranes also adds problems with tissue localization, even at the resolution of electron microscopy. In attempting to reconcile data suggesting that EAAT2 was present in both neurons and astrocytes, we went to some lengths before we could be sure of the distribution of this transporter. Localization to astrocytes was clearly evident from the distribution of immungold label over astrocyte membrane/profiles; but how could a low level of expression of EAAT2 be ruled in or out, since the membranes are close together

(Fig. 9)? Although much higher resolutions can be achieved by electron microscopy than by light or confocal microscopy, even for electron microscopy the lateral resolution of immunogold labeling (~28 nm with 15 nm gold particles, Matsubara *et al.*, 1996) means labeling on membranes within 40 – 50 nm or each other is ambiguous. One way around this was by embedding a suspension of synaptosomes where nerve terminals could be distinguished completely from glial membranes. A low level of expression in the nerve terminal membrane was then observed (Fig 9) (Furness *et al.*, 2008)).

[Figure 9 about here]

[Figure 10 about here]

Beware - convincing images are seductive

If the hypothesis is intriguing and the images appear to match it closely, it is easy to be carried away and accept the appearance at first sight (Roth, 2006). For instance, during the work to localize the EAAT3 glutamate transporter subtype (Holmseth *et al.*, 2012b) we wanted to test if we could reproduce the perisynaptic distribution observed by others (He *et al.*, 2001). To do this, post-embedding immuno-gold labeling was performed (Furness *et al.*, 2008). Very convincing images were obtained (Fig. 11). Afterwards (but before publication) we obtained EAAT3 knockout mice (Peghini *et al.*, 1997) and used them as negative controls. The same labeling pattern was seen. This was very disappointing and our reaction was accordingly: "*This cannot not be true! It must be something wrong with the knockout mouse!*" However, after more hard work testing the knockout mouse, we had to accept that we could not find anything wrong with it. The consequence was that the labeling pattern observed was an artifact. We had to scrap the data. This was a *double* knockout, indeed.

[Figure 11 about here]

Some thoughts about guidelines for immunocytochemistry

Since the field of immunocytochemistry is, as outlined above, still troubled by spurious results due to insufficient controls of antibody specificity, the arguments for improvements in quality control are strong. It is, however, hard to define the exact tests that should be performed. Although there are arguments for formal rules, we fear that such rules may cause problems. To improve quality and help advancing science, formal rules will have to be exact and very complex. Worse, they will be based on present knowledge. Because each project has unique challenges, but also opportunities, freedom is important. Skilled investigators will come up with smart solutions exploiting the opportunities and trying to circumvent the obstacles. Further, the demand for rigorous testing depends on the type and focus of each study. The testing has to be more rigorous if immunocytochemistry is the main focus than if it is a side issue. Similarly, testing can be less stringent if the data are confirmatory. On the other hand if the authors report that previous investigators have overlooked something, then the question is whether this is really the case or whether the antibodies in the new study cross-react with other molecules. Further, the technical difficulties involved need to be considered. It is for instance more difficult to localize proteins expressed at low levels than highly expressed proteins. Although highly sensitive techniques are available (e.g. tyramide signal amplification: Kerstens *et al.*, 1995; Silahtaroglu *et al.*, 2007), the specificity becomes more challenging as it becomes more likely that a cross-reacting molecular species might contribute more to the overall labeling than those under study.

To give room for novel approaches, perhaps it would be sufficient to ask the authors to explain in detail why they believe their labeling is specific. If the authors are pushed to explain the logic behind their conclusions, a judgment can be made whether this is good enough for the present purpose and situation. Because it is logically impossible to prove absolute specificity, some uncertainty must be acceptable. The degree of permissible uncertainty must be judged in each case. Testing is expensive and time-consuming. The following points may be addressed:

What is the evidence that the antibody recognizes the target antigen, and still does so after the treatment that the tissue has been subjected to?

Why is it likely that the labeling represents the antigen of interest and nothing else?

Does the tissue express the target antigen at detectable levels? Is the labeling intensity in reasonable agreement with expression levels? Multiple factors can affect the labeling intensity so definite conclusions can only be made if the antibody has been calibrated against known protein concentrations under identical conditions. However, strong labeling of sections despite low mRNA levels and weak or absent signal on Western blots are examples of mismatches that warrant extra specificity controls.

Does the labeling pattern in sections correlate with data from other methods, e.g. Western blots and *in situ* hybridization? For instance, if the labeling seen in sections is stronger in the hippocampus than in the cerebellum, then a similar difference is expected on immunoblots. Another example, if intact cells display very little, but still detectable glutamate uptake activity, then one or more glutamate transporting proteins must be present in their plasma membranes. If in this situation antibodies to relevant transporters fail to detect labeling in the plasma membranes, but give rise to strong labeling of mitochondria, then the most likely interpretation is that the

mitochondrial labeling is due to cross-reactivity and the absence of plasma membrane labeling is due to expression levels below the detection limit.

Can the tissue or subcellular localization be confused? Can specific structures be adequately isolated from each other microscopically to be certain where the labeling is distributed?

Quantitative considerations are necessary when discussing physiological roles. What can a given number of molecules accomplish?

In the case of protein antigens, the authors should state if relevant genetically modified organisms exist. If they do, and are not used as controls, then the authors should explain why.

Conclusions

High quality data on protein localization and expression levels are important as the biomedical research community relies on them directly or indirectly. Publication of misleading data happens too often and has down-stream consequences. Rather than introducing more formal rules and regulations, it may be more effective to simply ask authors to explain why they believe their data are valid. The perfect negative control is usually unavailable and the pre-absorption test can give a false impression of specificity. It is important to combine immunocytochemistry both with other methods and with quantitative considerations, and then see if all the pieces of information add up. Finally, when doubt about validity arises in a late phase of a project, there may not be any good solutions at all. If money has run out and a PhD is at stake, non-scientific factors will influence decisions. We hope that sharing our personal experiences will sensitize others to early warning signs and thereby facilitate navigation around some of the difficult situations we have found ourselves in, where

a lot of hard work and effort has turned out to have been wasted.

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FIGURE LEGENDS

Figure 1. Immunocytochemical labeling can always be obtained by adjusting assay conditions and cannot be used as proof of protein expression without a good negative control. Determination of optimal antibody concentrations is straightforward when tissues from knockout animals are available as negative controls. Sections from wild-type (WT) and EAAT3 (slc1a1)-deficient mice (KO) were incubated with either anti-C491 (Ab#371) or anti-C510 (Ab#565) antibodies to EAAT3 in concentrations as indicated. Note both that labeling is obtained in the EAAT3-deficient tissue at high antibody concentrations, and that the anti-C491 antibody cross-reacts with a non-EAAT3 epitope even at low antibody concentrations in some regions (arrowheads: hippocampus and striatum). Scale bar = 2 mm. (Reproduced from Holmseth *et al.*, 2012a, doi: 10.1369/0022155411434828).

Figure 2. The C-terminus of EAAT2 is degraded faster than the central portion of the protein. Mouse brain tissue that stored for the indicated time after death (for methods see: Li *et al.*, 2012), was subjected to Western blotting with the anti-B563 antibody (Ab#355; Holmseth *et al.*, 2009) to the extreme C-terminus (residues 563-573) of rat EAAT2 and with the anti-B146 antibodies (Cat. No. 250 203; Synaptic Systems GmbH, Goettingen, Germany, www.sysy.com) central parts (residues 146-161). Each lane contained 30 µg total protein. Note that most of the immunoreactivity detected with Anti-B563 is gone after 24 hours post-mortem (at room temperature) while there is still substantial immunoreactivity after 72 hours with the anti-B146 antibodies. Tissue extracts from the EAAT2 knockout mice (GLT1-KO; Tanaka *et al.*, 1997) were used as negative controls.

Figure 3. Confocal images of sections double labeled with anti-B563 antibodies (Ab#355; 0.1 µg/ml) to the C-terminus of EAAT2 and with anti-B493 antibodies to central parts of the protein (residues 493-508; Ab#8; 0.1 µg/ml; Li *et al.*, 2012). Note that there is co-localization in freshly fixed tissue (0 h), but not in tissue stored at room temperature for 24 h before fixation. Tissue from EAAT2 knockout mice (GLT1-KO; Tanaka *et al.*, 1997) was used as negative controls. Scale bar: 20 µm.

Figure 4. Oxidation of the EAAT2 makes higher molecular mass species appear on immunoblots (Trotti *et al.*, 1998; Danbolt, 2001). These bands, however, are not seen if reducing agents (e.g. DTT, dithiothreitol) are added. Note that EAAT2 is mostly in monomer form if homogenized directly in SDS (Lane 1). On the other hand, if exposed to an SH-group oxidizer (DTNB, 5-(3-carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid) oligomer bands appear (Lane 2) unless excess DTT is added prior to electrophoresis (Lane 4). PMSF (phenylmethanesulfonyl fluoride) was added to inhibit proteases and NaPi (sodium phosphate) was chosen as buffer. The blot was developed with 0.2 µg/ml anti-B12 antibody (Ab#360 Holmseth *et al.*, 2005).

Figure 5. Protein aggregation with unknown relationship to the native oligomeric structure occurs at elevated temperatures when solubilized with mild detergents (Danbolt, 2001). **Panel A:** Brain membranes (the water insoluble pellet produced after homogenization in water followed by centrifugation) have been incubated with increasing concentrations of crosslinker as indicated, solubilized in SDS with dithiothreitol (DTT), mixed with SDS-sample buffer (2 % SDS) and subjected to electrophoresis (7 % acrylamide) followed by immunoblotting with anti-EAAT2 antibodies (anti-B12 Ab#360; 0.2 µg/ml). Note that dimers and trimers are seen when

crosslinker is added. **Panel B:** Brain membranes were kept under reducing conditions (DTT added to all samples: Lanes 1-8). The membranes were solubilized in SDS (Lanes 1 and 2), cholate (Lanes 3-5) or Triton X-100 (Lanes 6-8). The extracts were either kept at room temperature (RT), at 0°C or at 37°C as indicated, and subjected to electrophoresis and immunoblotting as above. Note that monomers predominate in the SDS extracts. In the cholate and Triton extracts dimers and trimers are seen. The fraction of EAAT2 that runs as dimers or trimers increases if the extracts have been incubated at elevated temperatures. NB: Note that these dimers and trimers do not dissolve in the SDS sample buffer. **Both panels:** Proteases were inhibited with 5 mM EDTA (ethylenediaminetetraacetic acid) and 1 mM PMSF (phenylmethylsulfonyl fluoride).

Figure 6: Non-EAAT2 proteins interfere with EAAT2 immunodetection possibly by competition for binding sites on the nitrocellulose membrane. **Panel A:** application of increasing amounts 1.25, 2.5 and 5 µl of SDS solubilized transfected HEK293T cells leads to increased EAAT2 immunoreactivity on the blots. **Panel B:** SDS solubilized adult rat hippocampus (HC, 1 µg total tissue protein) was mixed with increasing amounts of SDS solubilized un-transfected HEK293T cells. Lanes 1-4 contain, respectively, (about 3, 9 or 30 µg total HEK293T cell protein. Note that the detection of hippocampal EAAT2 decreases with increasing amounts of cell extract. EAAT2 was detected with anti-EAAT2a antibodies (Ab#355: 0.1 µg/ml) followed by HRP-conjugated secondary antibodies. This figure is based on materials produced previously (Holmseth *et al.*, 2009).

Figure 7. Antibodies to EAAT2 cross-react with a pancreatic water soluble protein (Zhou *et al.*, 2014b). Pancreas from wildtype (WT) and EAAT2 knockout (KO) mice (Tanaka *et al.*, 1997) were homogenized in water with protease inhibitors and centrifuged to separate the water soluble proteins (supernatant) from membrane proteins (pellet). Both the pellets and the supernatants were mixed with SDS-sample buffer and Western blotted with anti-B493 antibodies (Ab#95; 0.1 µg/ml) to EAAT2. The protein loading was first verified by Ponceau S staining (A), then destained and probed with the antibodies (B). Note that something with an electrophoretic mobility similar to EAAT2 was labeled, but that this was in the supernatants and from both WT and KO animals. There was no labeling of the membrane proteins. Spinal cord samples (C) were run for comparison. This was on the same electrophoresis gel and blot, but the blot was cut to hide lanes that were irrelevant in this context. Similar results were obtained with the anti-B12 antibody (Ab#360) to EAAT2 (not shown). Conclusion, EAAT2 could not be detected in pancreas.

Figure 8. A high magnification transmission electron micrograph showing an asymmetric synapse in the cochlear nucleus of a guinea pig (section courtesy of Dr S Mahendrasingam, image by DNF). An axon terminal (T) with a mitochondrion (m) and multiple synaptic vesicles (sv) is synapsing onto a dendritic spine (S). The postsynaptic density (PSD) is the darker zone in the middle. The synaptic cleft is the narrow lighter line between the two arrowheads. Note the large amounts of plasma membranes. At least 10, possibly 11, different cellular compartments (cellular extensions) can be identified in the image. They are all separated by extracellular space bordered by the plasma membranes of neighboring cells. However, the membranes cannot be seen clearly in places where the membrane surface is oblique

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Figure 10. **Panel A:** Post-embedding immunogold labeling for EAAT2 on a hippocampal slice. Two terminals (T), an associated spine (Sp) and glial process (G) are visible. Gold-labeling is clearly predominant in the glial process, but there are ambiguous particles that could be localized either to the terminal membrane or the membrane of glial process (arrows). **Panel B:** In a synaptosome preparation, isolated terminals (T) are separated from the associated glial membranes (G) and so unambiguous identification of terminal membrane labeling becomes possible (arrows). Scale bar = 200 nm.

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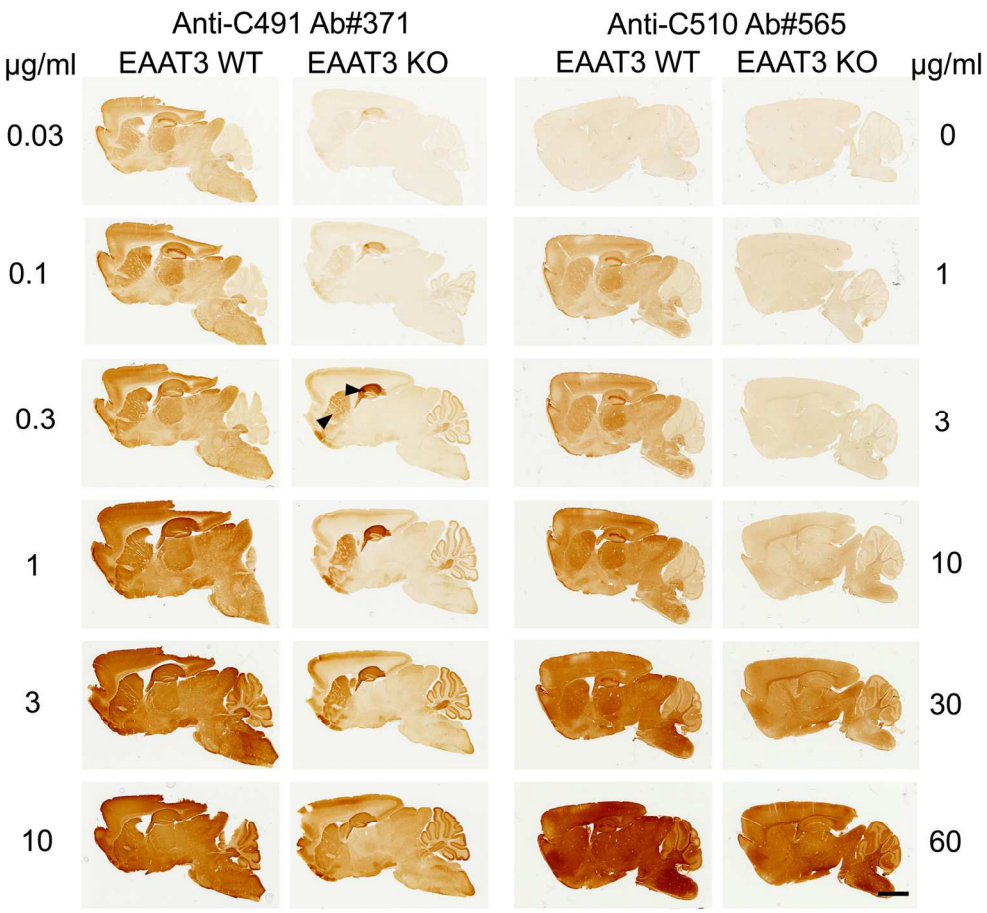


Figure 1. Immunocytochemical labeling can always be obtained by adjusting assay conditions and cannot be used as proof of protein expression without a good negative control. Determination of optimal antibody concentrations is straightforward when tissues from knockout animals are available as negative controls. Sections from wild-type (WT) and EAAT3 (slc1a1)-deficient mice (KO) were incubated with either anti-C491 (Ab#371) or anti-C510 (Ab#565) antibodies to EAAT3 in concentrations as indicated. Note both that labeling is obtained in the EAAT3-deficient tissue at high antibody concentrations, and that the anti-C491 antibody cross-reacts with a non-EAAT3 epitope even at low antibody concentrations in some regions (arrowheads: hippocampus and striatum). Scale bar = 2 mm. (Reproduced from Holmseth et al., 2012a, doi: 10.1369/0022155411434828).
154x142mm (300 x 300 DPI)

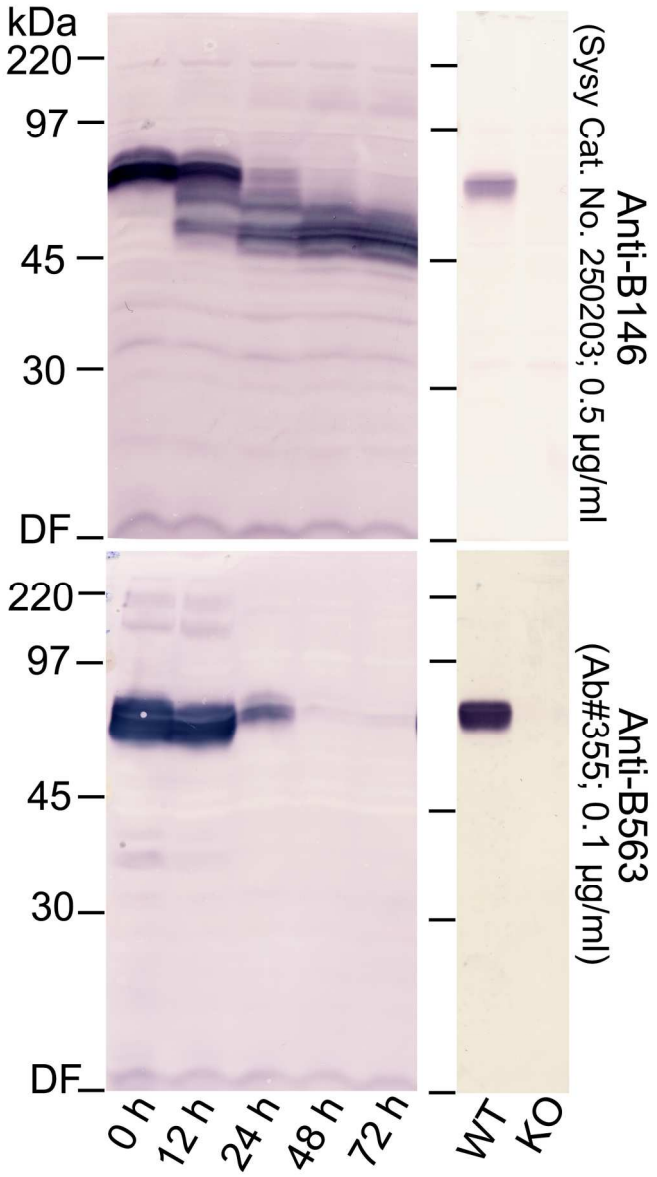


Figure 2. The C-terminus of EAAT2 degrade faster than the central portion of the protein. Mouse brain tissue that stored for the indicated time after death (for methods see: Li et al., 2012), was subjected to Western blotting with the anti-B563 antibody (Ab#355; Holmseth et al., 2009) to the extreme C-terminus (residues 563-573) of rat EAAT2 and with the anti-B146 antibodies (Cat. No. 250 203; Synaptic Systems GmbH, Goettingen, Germany, www.sysy.com) central parts (residues 146-161). Each lane contained 30 µg total protein. Note that most of the immunoreactivity detected with Anti-B563 is gone after 24 hours post-mortem (at room temperature) while there is still substantial immunoreactivity after 72 hours with the anti-B146 antibodies. Tissue extracts from the EAAT2 knockout mice (GLT1-KO; Tanaka et al., 1997) were used as negative controls.
126x226mm (300 x 300 DPI)

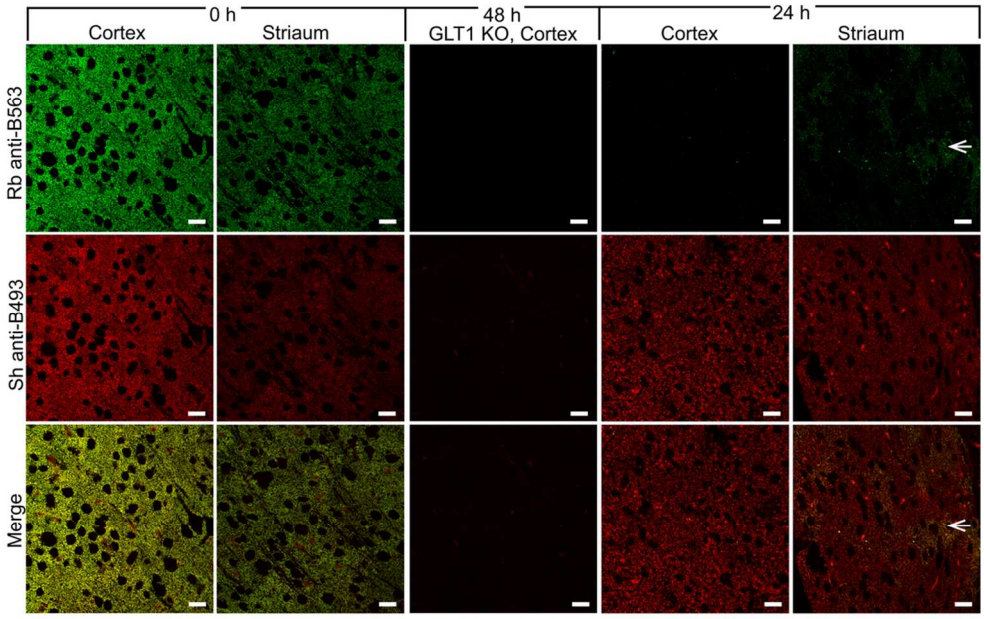


Figure 3. Confocal images of sections double labeled with anti-B563 antibodies (Ab#355; 0.1 µg/ml) to the C-terminus of EAAT2 and with anti-B493 antibodies to central parts of the protein (residues 493-508; Ab#8; 0.1 µg/ml; Li et al., 2012). Note that there is co-localization in freshly fixed tissue (0 h), but not in tissue stored at room temperature for 24 h before fixation. Tissue from EAAT2 knockout mice (GLT1-KO; Tanaka et al., 1997) was used as negative controls. Scale bar: 20 µm.
106x67mm (300 x 300 DPI)

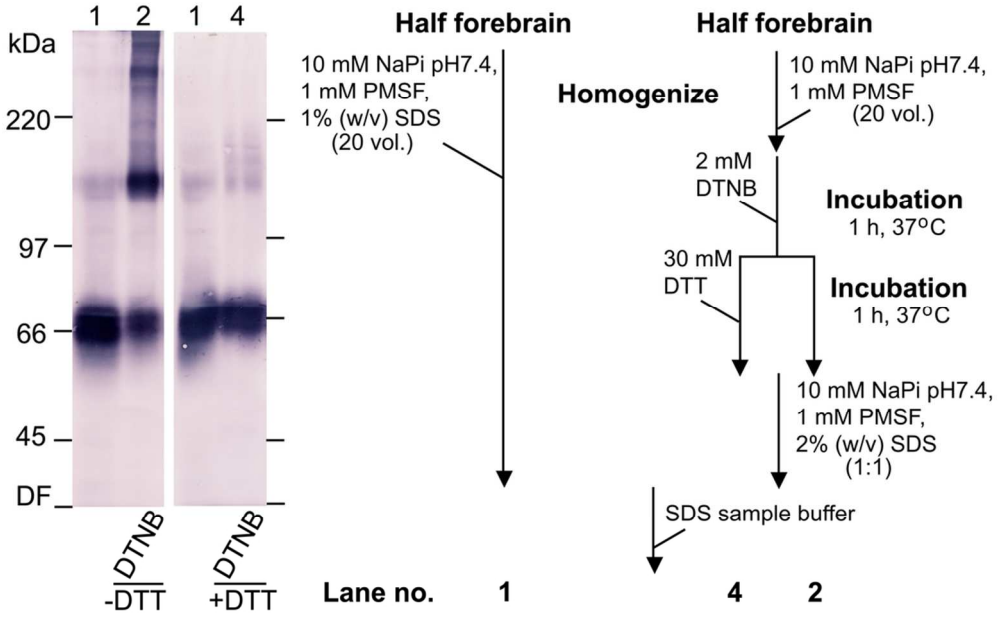


Figure 4. Oxidation of the EAAT2 makes higher molecular mass species appear on immunoblots (Trotti et al., 1998; Danbolt, 2001). These bands, however, are not seen if reducing agents (e.g. DTT, dithiothreitol) are added. Note that EAAT2 is mostly in monomer form if homogenized directly in SDS (Lane 1). On the other hand, if exposed to an SH-group oxidizer (DTNB, 5-(3-carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid) oligomer bands appear (Lane 2) unless excess DTT is added prior to electrophoresis (Lane 4). PMSF (phenylmethanesulfonyl fluoride) was added to inhibit proteases and NaPi (sodium phosphate) was chosen as buffer. The blot was developed with 0.2 µg/ml anti-B12 antibody (Ab#360 Holmseth et al., 2005).
98x61mm (300 x 300 DPI)

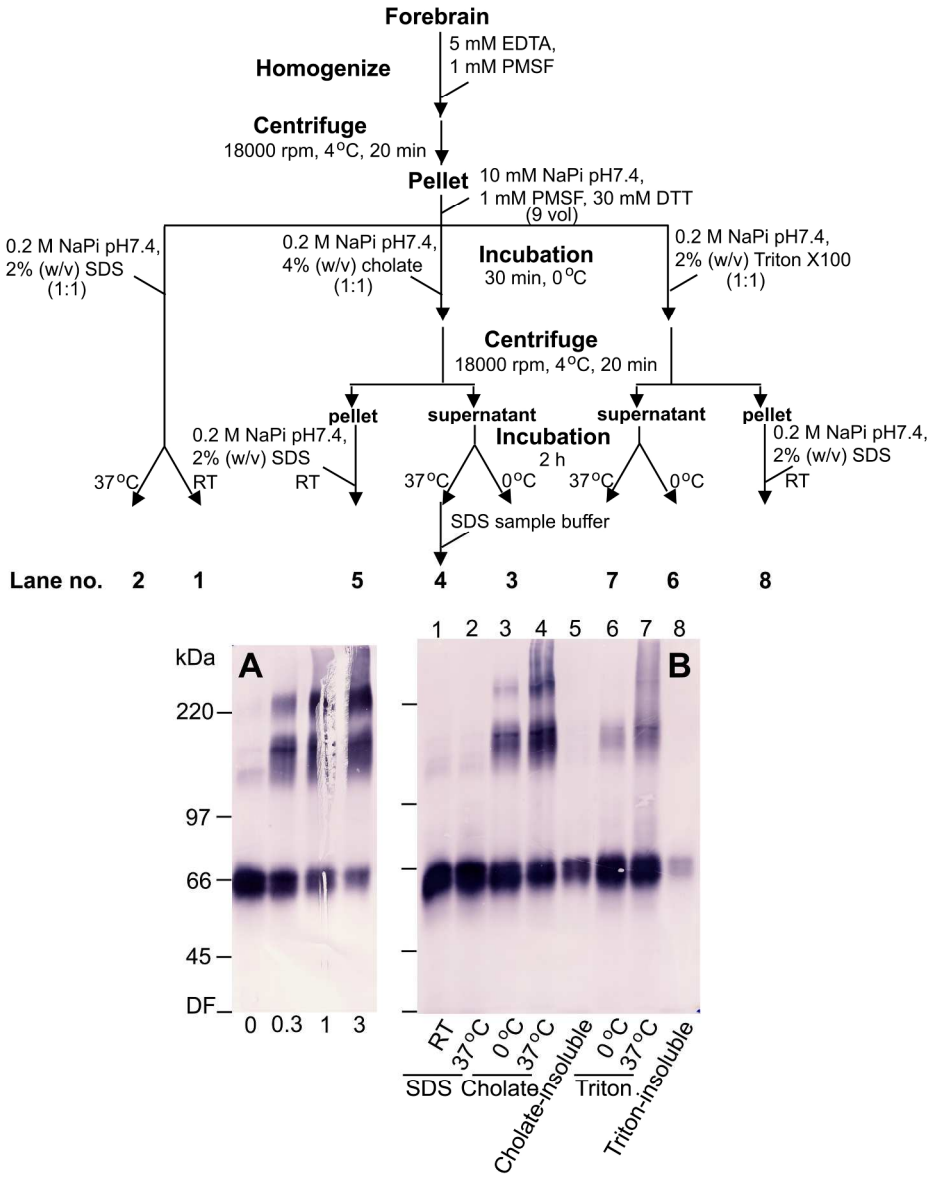


Figure 5. Protein aggregation with unknown relationship to the native oligomeric structure occurs at elevated temperatures when solubilized with mild detergents (Danbolt, 2001). Panel A: Brain membranes (the water insoluble pellet produced after homogenization in water followed by centrifugation) have been incubated with increasing concentrations of crosslinker as indicated, solubilized in SDS with dithiothreitol (DTT), mixed with SDS-sample buffer (2 % SDS) and subjected to electrophoresis (7 % acrylamide) followed by immunoblotting with anti-EAAT2 antibodies (anti-B12 Ab#360; 0.2 µg/ml). Note that dimers and trimers are seen when crosslinker is added. Panel B: Brain membranes were kept under reducing conditions (DTT added to all samples: Lanes 1-8). The membranes were solubilized in SDS (Lanes 1 and 2), cholate (Lanes 3-5) or Triton X-100 (Lanes 6-8). The extracts were either kept at room temperature (RT), at 0°C or at 37°C as indicated, and subjected to electrophoresis and immunoblotting as above. Note that monomers predominate in the SDS extracts. In the cholate and Triton extracts dimers and trimers are seen. The fraction of EAAT2 that runs as dimers or trimers increases if the extracts have been incubated at elevated temperatures. NB: Note that these dimers and trimers do not dissolve in the SDS sample buffer. Both panels: Proteases were

inhibited with 5 mM EDTA (ethylenediaminetetraacetic acid) and 1 mM PMSF (phenylmethylsulfonyl fluoride).
223x285mm (300 x 300 DPI)

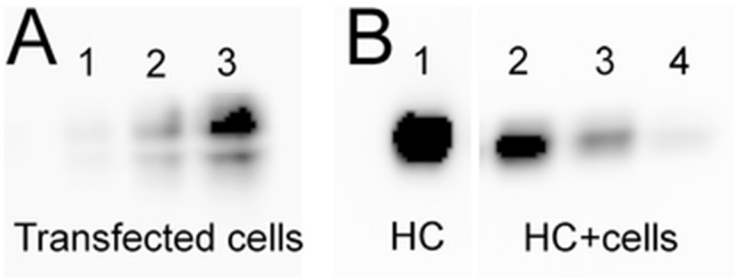
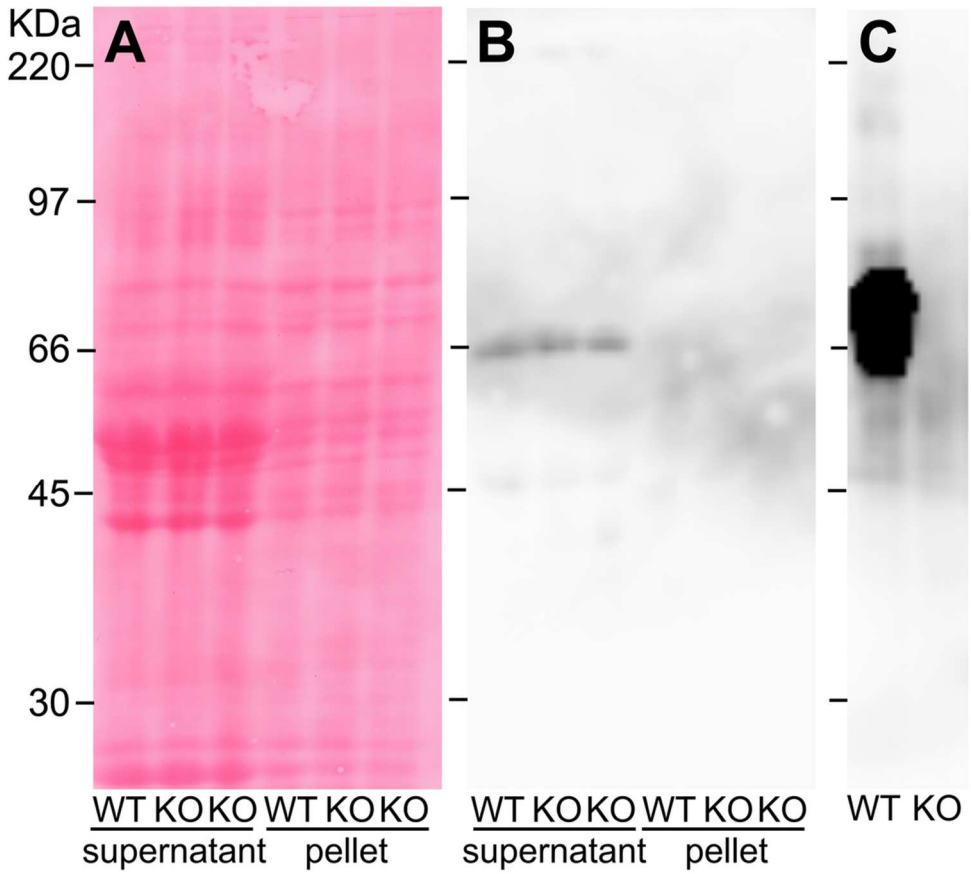


Figure 6: Non-EAAT2 proteins interfere with EAAT2 immunodetection possibly by competition for binding sites on the nitrocellulose membrane. Panel A: application of increasing amounts 1.25, 2.5 and 5 µl of SDS solubilized transfected HEK293T cells leads to increased EAAT2 immunoreactivity on the blots. Panel B: SDS solubilized adult rat hippocampus (HC, 1 µg total tissue protein) was mixed with increasing amounts of SDS solubilized un-transfected HEK293T cells. Lanes 1-4 contain, respectively, (about 3, 9 or 30 µg total HEK293T cell protein. Note that the detection of hippocampal EAAT2 decreases with increasing amounts of cell extract. EAAT2 was detected with anti-EAAT2a antibodies (Ab#355: 0.1 µg/ml) followed by HRP-conjugated secondary antibodies. This figure is based on materials produced previously (Holmseth et al., 2009).
31x12mm (300 x 300 DPI)



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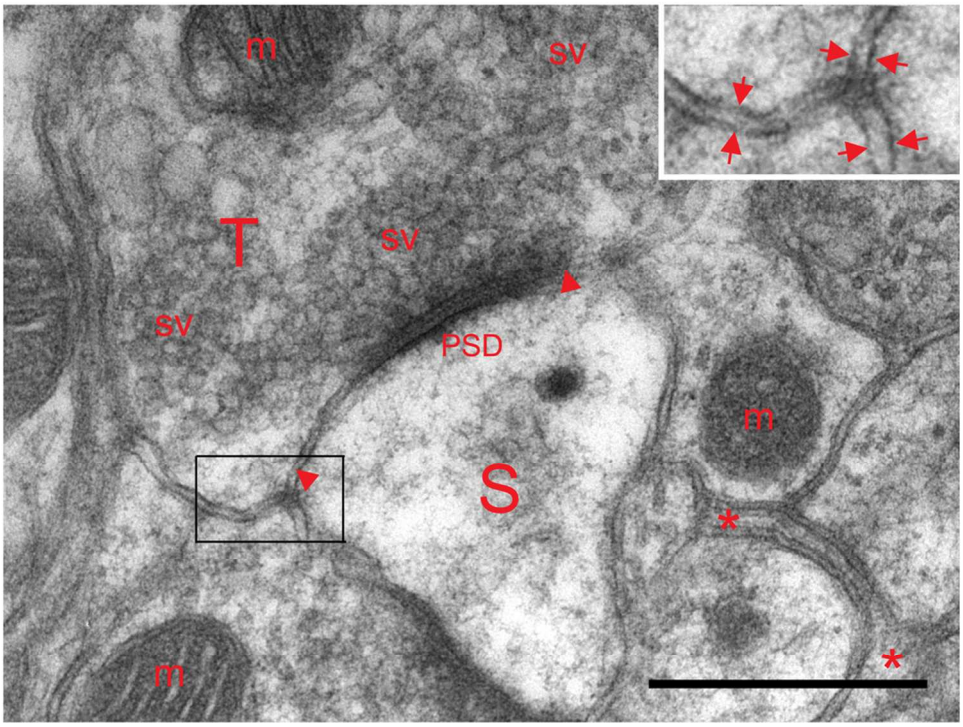
99x89mm (300 x 300 DPI)



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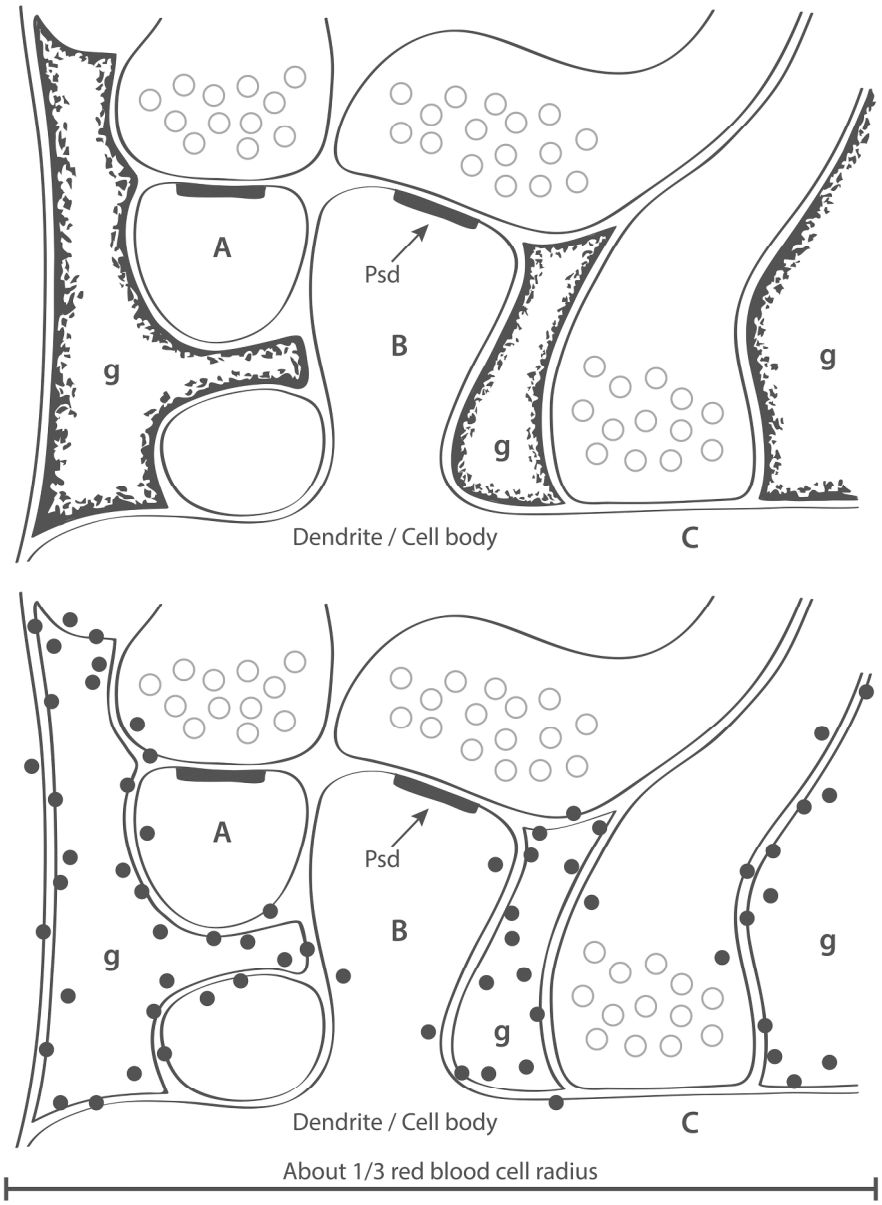
97x75mm (300 x 300 DPI)



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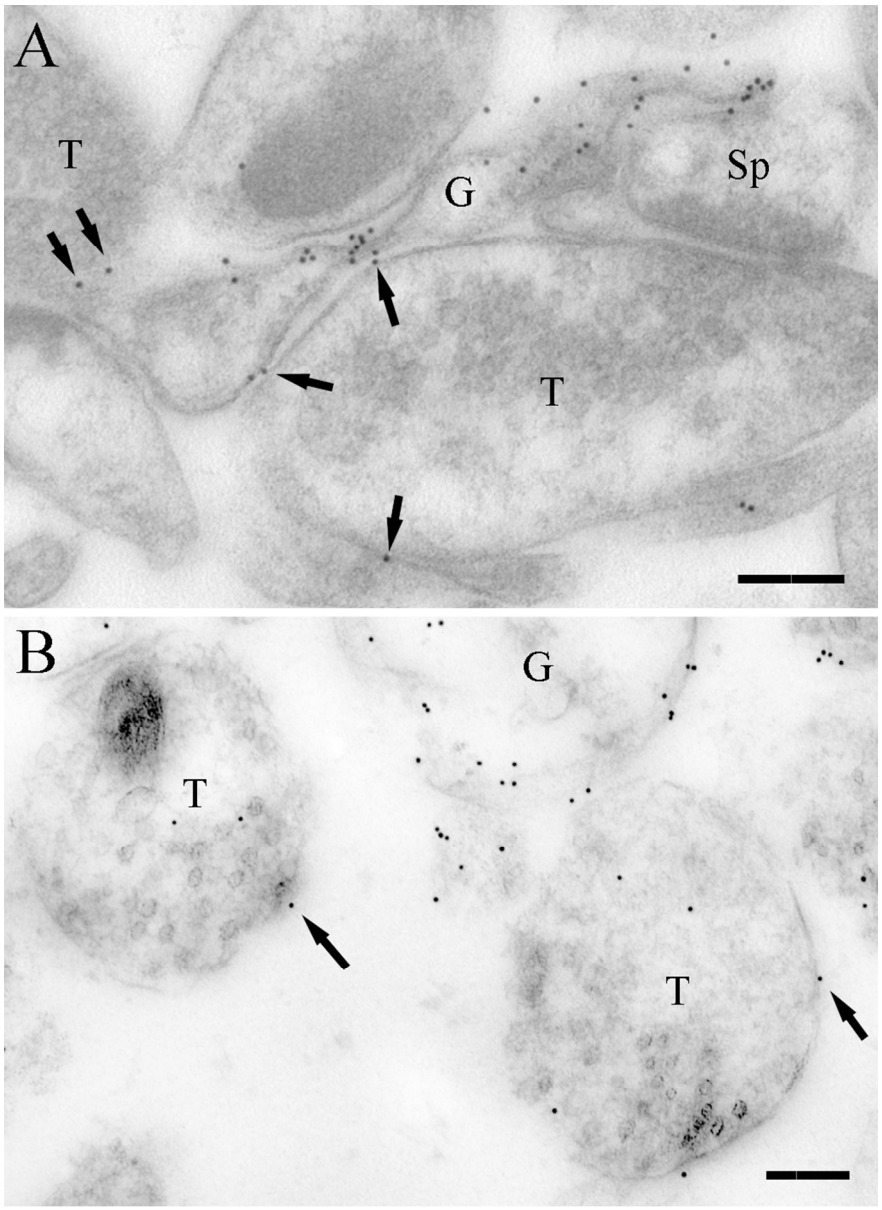

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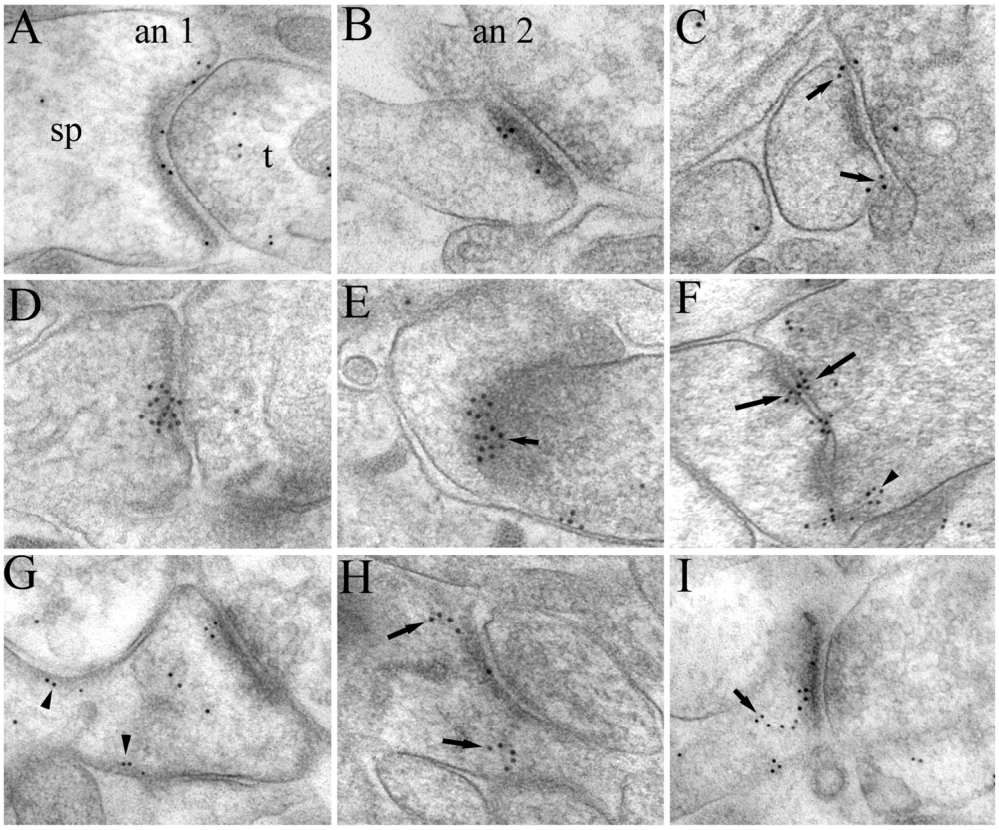
87x119mm (300 x 300 DPI)



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142x118mm (300 x 300 DPI)

Table 1

Source	Web
Allan Brain Atlas	http://connectivity.brain-map.org/transgenic/search/basic
International Knockout Mouse Consortium	www.knockoutmouse.org
Mouse Genome Informatics	www.informatics.jax.org
NIH Mouse Initiatives - The Knockout Mouse Project	http://www.genome.gov/17515708
Gene Expression Nervous System Atlas (GENSAT)	http://www.gensat.org/daily_showcase.jsp
Cre-lines and Bac-mice	http://www.gensat.org/cre.jsp http://cre.jax.org/data.html
International Mouse Phenotyping Consortium	http://www.mousephenotype.org/data/search
Mutant Mouse Regional Resource Centers (MMRRC	https://www.mmrrc.org/index.php

A large number of modified animals are available. Before starting a knockout or conditional knockout project, it is a good idea to check if the gene of interest has been targeted and the availability of the targeting vector/ES cell clones/mice.